PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 11/04, 11/14	A1	**(11) International Publication Number:** WO 87/ 02704 **(43) International Publication Date:** 7 May 1987 (07.05.87)

(21) International Application Number: PCT/GB86/00644

(22) International Filing Date: 21 October 1986 (21.10.86)

(31) Priority Application Numbers: 8526095
8616881

(32) Priority Dates: 22 October 1985 (22.10.85)
10 July 1986 (10.07.86)

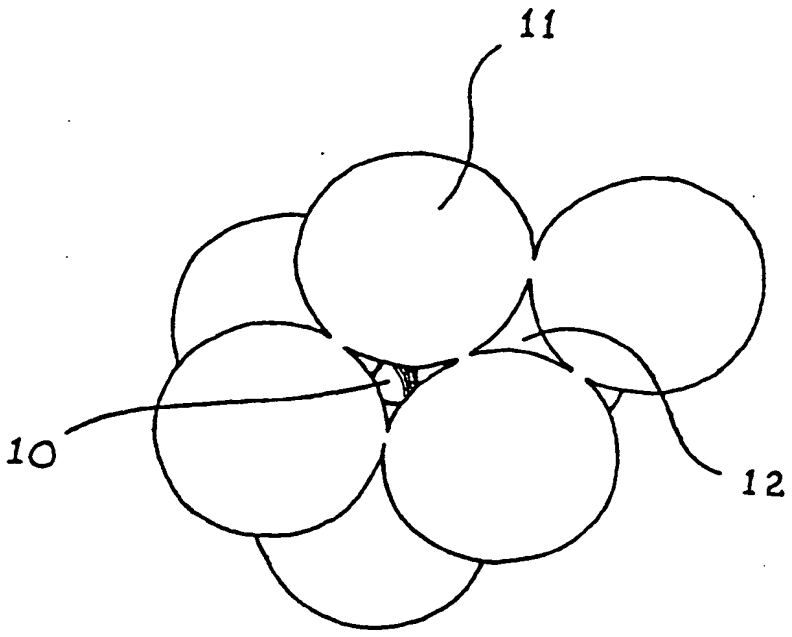
(33) Priority Country: GB

(71)(72) Applicant and Inventor: ROBINSON, Eric [GB/GB]; 146 Moss Road, Lambeg, Lisburn, Antrim BT27 4LF (GB).

(81) Designated States: BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: PROCESS FOR CELL IMMOBILISATION



(57) Abstract

A process for immobilising cells in a structured matrix engineered to suit the cell, producing a strong porous support for the cells which has good mass transfer characteristics. Cells are entrapped in cavities between microparticles which are bonded at points of contact to construct the rigid matrix.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BG	Bulgaria
BJ	Benin
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

PROCESS FOR CELL IMMOBILISATION

The present invention relates to entrapment of materials, for example viable cells, within a solid framework.

The use of immobilised microbial, plant or animal cells for the production of pharmaceutical products and fine chemicals is gaining in commercial importance. The advantages of the use of immobilised cells include repeated use, continuous process operation and the elimination of the separation steps necessary if free cells are used which must be removed from product solutions.

Amongst the various methods devised for immobilising cells, entrapment within polymer gels, for example polyacrylamide gels, has been most extensively applied. A disadvantage of existing systems is that to obtain adequate strength the gel structure must be dense and this reduces the porosity of the system. Mass transfer within the immobilising matrix is thus limited.

The object of the present invention is to provide an improved process for cell immobilisation which overcomes this difficulty, producing a strong porous support matrix for the cells which has good mass transfer characteristics.

According to the present invention there is provided a process for immobilising cells which comprises the steps of mixing the cells with microparticles which are substantially insoluble in aqueous media, blending thoroughly to uniformly disperse the cells and microparticles then bonding the microparticles at points of contact to form a permanent porous matrix entrapping the cells within the cavities therein.

It is preferred but not required that the microparticles are spherical in shape and uniform in diameter. Where uniform microspheres are used their diameter should be less than six times the smallest dimension of the cell.

The microparticles used may be composed of inorganic oxides, hydroxides, carbonates or sulphates which are substantially insoluble in an aqueous medium such as silica, alumina, aluminium hydroxide, calcium carbonate or calcium sulphate or of an organic polymer such as polyacrylamide, polystyrene, polyvinyl chloride, polyvinyl acetate, dextran, cellulose or starch. Mass transfer through the matrix is improved if the microparticles are themselves porous.

Normally the microparticles and cells are mixed and blended in water or in a medium compatible with the viability of the cells.

Particularly suitable microparticles are silica microspheres produced according to my copending International Application No. PCT/GB86/00319. Microspheres of the appropriate size held in aqueous slurry may be close packed and bonded at points of contact by dewatering and drying. Cells mixed homogeneously with these microspheres before dewatering are entrapped within the cavities in this matrix. Dewatering is achieved by aspiration or by the application of pressure after which the mass is dried in a current of air at 20° to 30°C.

Where the microparticles do not bond naturally they may be precoated with an adhesive which softens in the medium employed for mixing and dispersing the cells and microparticles.

Preferably the microparticle should not be more than six times larger than the cells and ideally not more than three

times the smallest dimension of the cells otherwise cells may escape from the matrix by moving through the interstitial pores.

After the cells and microparticles are mixed and before the microparticles are finally bonded together the plastic mass may be formed into any required shape and size by for example extrusion, by pressing into pellets or molding into beads or tablets.

The means whereby cells are entrapped within the matrix of microparticles will be further apparent from the accompanying drawings of preferred embodiments of the invention. Figure 1 illustrates a small element of an entrapment matrix. A cell 10 is entrapped in the cavity formed by six close packed spherical microparticles 11. The ratio of the diameter of the microparticle to that of the cell is greater than 2.5:1.0 but no more than 6.0:1.0 otherwise cells will escape through the interstitial pores 12 between cavities. Figure 2 illustrates in cross-section a small element of an entrapment matrix in which a larger cell 13 creates a cavity by replacing a microparticle 14 in the matrix. Here microparticles similar in size to the cells are used. In the first case access to the entrapped cell is by way of six interstitial pores 12 through which nutrients, expressed proteins or gases may pass. In the second case access to the cell is gained through at least 24 such interstitial pores.

The strength of the porous matrix may be improved by treatment with a solution of a polymer, or a solution of a monomer which can be polymerised, to form a porous skin or coating around the package of cells and microparticles. The treatment is carried out after shaping into beads, pellets or lumps which are immersed in a solution of a polymer in an appropriate solvent such that at least part of the solution is taken up by the entrapment matrix after which the excess solution is removed.

The polymer used may be for example cellulose acetate, polystyrene, polyacrylonitrile, polyacrylamide, polyamide or polyvinylchloride. Where the polymer is applied in a solvent, appropriate solvents include acetone, chloroform or dimethylformamide. The solvent chosen should not affect the immobilising matrix. The strength of the polymer solution used may be from 0.1% to 20% and is preferably from 1% to 5%.

Alternatively the package of cells and microparticles may be treated with a solution of a monomer which is subsequently polymerised. Thus for example beads or pellets may be treated with a solution of sebacoyl chloride in chloroform. These are then transferred to an aqueous solution of hexamethylene diamine to form a skin of polyamide around the beads or pellets.

The benefits of forming a porous skin or coating within the outer layers of the matrix containing immobilised cells are twofold. First it acts by tying in cells exposed on the surface which otherwise break free from the immobilising matrix. Second it adds strength to the package, in particular it improves the retention of strength over long periods of use.

The skin or coating may be applied before or after drying the matrix holding entrapped cells. In addition it may be semi-permeable.

The invention will be further apparent from the following examples.

EXAMPLE 1

Cells of Saccharomyces cerevisiae were mixed with silica microspheres 7 μm in diameter and the mixture was carefully blended for 15 minutes. The semi fluid mass was transferred

to a vessel fitted with an oriface 3mm in diameter. The vessel was vibrated to fluidise the mixture which formed droplets as it passed through the oriface and fell onto an absorbant surface which aspirated interstitial water leaving moist beads which were dried in a current of air at 20°C. After drying the 4mm beads are hard and strong with a crushing strength of 0.75 Kg. The porosity was 0.5 ml/ml and the cell density of S. cerevisiae was approximately 3 x 10^9cells/cm^3.

The beads were packed into a fixed bed reactor of approximately 1 l capacity through which was pumped a solution containing 200 g/l sucrose at 30°C. The conversion of sucrose into ethanol was found to be 8g ethanol/l/hour.

EXAMPLE 2

Cells of S. cerevisiae were mixed and blended carefully with silica microspheres 7 µm in diameter for 15 minutes in aqueous slurry. The fluid mass was spread on filter paper to remove interstitial water after which the mass was dried at 25°C. The mass was broken and sieved through a 4 mesh sieve. The cell density within the matrix was approximately 5 x 10^9 cells/cm^3.

The material was packed into a fixed bed reactor of approximately 1 l capacity through which was pumped a solution containing 200 g/l sucrose. The conversion of sucrose to ethanol was found to be 10g ethanol/l/hour.

EXAMPLE 3

20g of 4 mm beads made from silica microspheres and containing 2g of yeast cells held in the interstitial cavities was treated with 50ml 4% polystyrene in chloroform for 2 minutes. The beads were removed and air dried for 48 hours at 20°C.

These beads were used in a continuous fermentation system behaving as an active biocatalyst for 4 months. The production of ethanol from sucrose was measured at 5g/l/hour.

EXAMPLE 4

Cells of aspergillus niger were mixed carefully with a neutral slurry of 3 μm silica microspheres and the mass was spread on filter paper to remove excess water. The mass was allowed to dry for 2 days in a current of air at 20°C, before being broken and sieved through a 4 mesh sieve.

When packed in a column through which a nutrient liquor was circulated the cells were observed to express a mixture of enzymes including pectinase, cellulase, and hemicellulase.

EXAMPLE 5

Spores of Phytophthora infestans were mixed thoroughly with 8 μm silica microspheres and the mass was formed into beads. These were dried at 25°C for 2 days. The beads were then moistened with a nutrient solution and held at 25°C for a further 7 days after which each bead was found to contain a mycelium.

When the beads were packed in a column and had a nutrient liquor circulated over them the immobilised microorganism produced cellulase.

EXAMPLE 6

20 g of 2mm diameter beads made from 7 μm silica microspheres and holding 3 g yeast cells in interstitial cavities were

treated for 2 minutes with 50 ml of a 2% solution of cellulose acetate in acetone before the beads were completely dried. The The beads were separated from the solution and the solvent removed before they were used in a batch fermentation to produce an aqueous ethanol solution.

EXAMPLE 7

1.7g S. cerevisiae was mixed with 30g 7µm particles of calciumsulphate hemihydrate in 20 ml water. Cells and microparticles were dispersed carefully and the mixture pressed into 4mm diameter tablet molds.

When set and dried the tablets were used to ferment a sucrose malt solution to a beer containing approximately 4% ethanol.

EXAMPLE 8

10g of alumina with a spheroidal particle shape approximately 7 µm across were added to 25ml of 8% polystyrene in chloroform. All the polystyrene solution was taken up by the microparticles which were kept in motion while the chloroform was removed. 1g yeast was suspended in 2.5ml water and this was added to 25ml acetone. The polystyrene coated alumina was added and the cells and alumina worked together for 5 mins. The paste was then pressed at 12 x 10^4 Pa to expel excess water/acetone The softened polystyrene coating behaved as an adhesive bonding the microparticles to entrap the yeast. The entrapped cells were used to ferment a glucose solution.

The method demonstrated by this example has been used successfully to form entrapment matrices using other inorganic microparticles and microspheres of organic polymers.

CLAIMS

1. A process for immobilising cells which comprises the steps of mixing the cells with microparticles which are substantially insoluble in aqueous media, blending thoroughly to uniformly disperse the cells and microparticles then bonding the microparticles at points of contact to form a permanent porous matrix entrapping the cells within the cavities therein.

2. A process as claimed in claim 1 wherein the microparticles are spherical in shape.

3. A process as claimed in claim 1 wherein the microparticles are uniform in diameter.

4 A process as claimed in claim 1 wherein the diameter of the microparticles used is less than six times the smallest dimension of the cell.

5. A process as claimed in claim 1 wherein the microparticles are composed of inorganic oxides, hydroxides,carbonates or sulphates which are substantially insoluble in aqueous media.

6. A process as claimed in claim 1 wherein the microparticles are composed of silica, alumina, aluminium hydroxide, calcium carbonate or calcium sulphate.

7. A process as claimed in claim 1 wherein the microparticles are composed of a polymer.

8. A process as claimed in claim 1 wherein the microparticles are composed of polyacrylamide, polystyrene, polyvinyl chloride, polyvinyl acetate, dextran, cellulose or starch.

9. A process as claimed in claim 1 wherein the microparticles and cells are mixed and blended in a liquid medium compatible with the viability of the cells.

10. A process as claimed in claim 1 wherein the microparticles are pretreated with an adhesive.

11. A process as claimed in claim 1 wherein the microparticles and cells are mixed and blended in a medium which softens an adhesive applied to the microparticles.

12. A matrix containing immobilised cells prepared by the process as claimed in any preceding claim.

13. A matrix as claimed in claim 12 which has been treated with a polymer solution.

14. A matrix as claimed in claim 12 which has been treated with a solution of cellulose acetate, polystyrene, polyamide, or polyvinyl chloride.

15. A matrix as claimed in claim 12 which has a polymer coating deposited thereon.

16. A matrix as claimed in claim 15 which has a semi-permeable coating deposited thereon.

PROCESS FOR CELL IMMOBILISATION

A process for immobilising cells in a structured matrix engineered to suit the cell, producing a strong porous support for the cells which has good mass transfer characteristics. Cells are entrapped in cavities between microparticles which are bonded at points of contact to construct the rigid matrix.

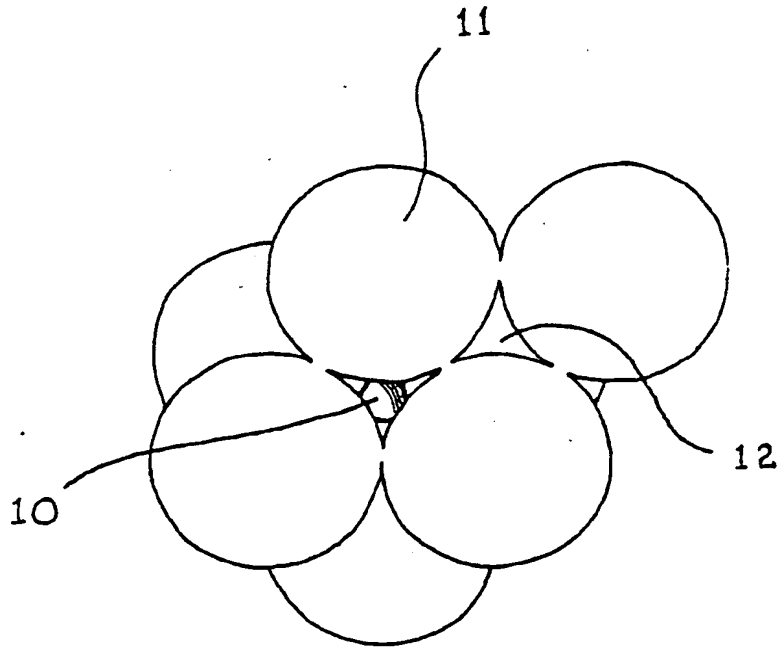


FIG. 1

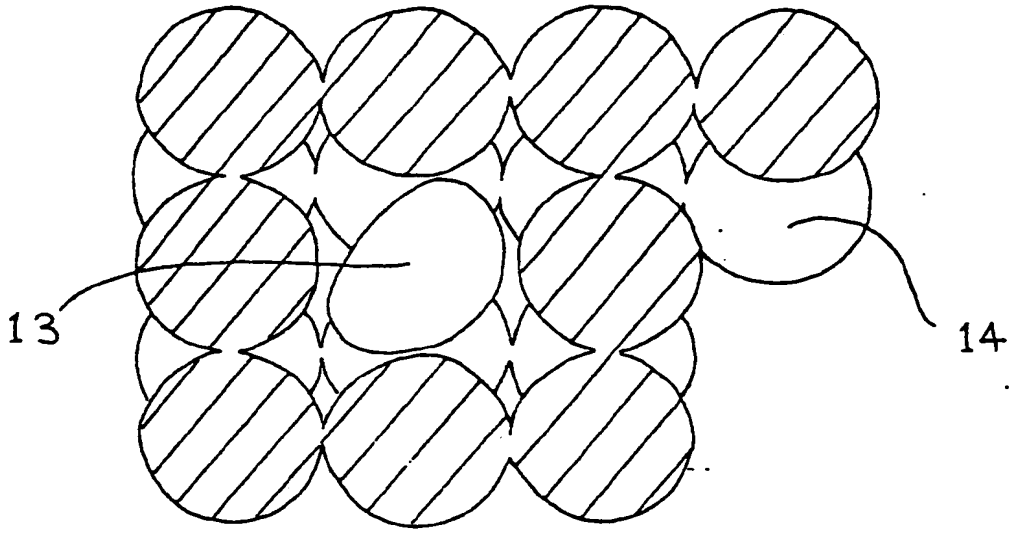


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00644

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 11/04; C 12 N 11/14

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0017176 (BASF) 15 October 1980, see claim; example 4; page 7, lines 23-34; page 8, lines 1-5; page 9, lines 3-8 --	1,3,5,6,9, 10,12,13
X	US, A, 4266029 (BRANNER-JØRGENSEN) 5 May 1981, see claims 1,2,4,13,20 --	1,5,6,10,12, 13,15
A	FR, A, 2160661 (UNILEVER) 29 June 1973, see claims 1,14,15	1,7,9,10, 12,13,15

- Special categories of cited documents: [10]
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th January 1987	19 FEV. 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

See notes on accompanying sheet

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00644 (SA 14986)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 05/02/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0017176	15/10/80	DE-A-	2912827	09/10/80
		JP-A-	55131393	13/10/80
		AT-B-	E4058	15/07/83
US-A- 4266029	05/05/81	NL-A-	7906064	18/02/80
FR-A- 2160661	29/06/73	GB-A-	1415301	26/11/75

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82